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13. ABSTRACT *(Maximum 200 Words)*

One of the most potent inhibitors of epithelial growth is TGFβ. Understanding the connection between TGFβ and cell cycle control is an important avenue of experimentation towards treating breast cancers. We are utilizing two complementary approaches in *C elegans* to find cell cycle regulatory genes that respond to TGFβ. First, we are using a cell cycle reporter, *ribonucleotide reductase*, to monitor cell cycle activation in mutagenized animals that are arrested at the dauer stage (a TGFβ induced developmental stage). Appropriate genetic strains have been constructed and tested, which will allow screening to proceed shortly. In a second approach, we are probing DNA microarrays with mRNAs collected from animals entering dauer. This experiment should identify genes whose regulation is altered by TGFβ as the animals undergo cell cycle arrest. During the next year, we hope to have isolated mutants from our screen and to have begun the molecular characterization of them.

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INTRODUCTION

More than 80% of breast cancers are composed of epithelial cells. One of the most potentially fruitful avenues to control breast cancers is to learn how to control the growth of epithelial cells. One of the most potent inhibitors of epithelial cell growth is TGFβ. TGFβ signaling plays a major role in the normal development of the breast and in the progression of breast cancers by controlling exit from the cell cycle. Thus the regulation of cell cycle exit constitutes a promising avenue towards treating breast cancers (Catzavelos *et al.*, 1997).

We are utilizing two complementary approaches in *C. elegans* to find cell cycle regulatory genes that respond to TGFβ signaling. First, in genetic screens using a cell cycle reporter gene, *ribonucleotide reductase*, we will look for mutations in loci that release dauer animals (a TGFβ induced developmental stage) from their cell cycle arrest (Hong *et al.*, 1998). Many of these genes may be directly regulated by the TGFβ pathway. Secondly, we are taking a complementary molecular approach to find genes regulated by the *C. elegans* TGFβ pathway. Using RNA from arrested animals and from animals released from dauer arrest, we have probed DNA microarrays containing the 17,700 genes (of 19.000 genes known) of *C. elegans* to identify ones whose expression is altered as animals are released from TGFβ induced arrest. These two approaches should provide us with candidate genes that connect the TGFβ pathway with specific novel, regulators of cell cycle arrest. To extend our findings, we will look in mammals for homologs of genes we discover in our experiments. This information will increase our understanding of how cell cycle regulation is achieved, and provide reagents for the design of novel therapeutics.

BODY

Task #1. During the last year, we reported that we had constructed a new *rnr::gfp* reporter. This was prompted because the original strain had unusual genetic properties and the intensity of the reporter was marginal. We duplicated the promoter region of the *C. elegans* ribonucleotide reductase gene (*rnr*) and inserted it into an appropriate vector for transformation into animals. The rationale for duplicating the promoter region is that this often results in higher expression levels.

We examined the expression levels of constructs containing two or three duplicated promoter regions to find the most intense. One line, containing two copies of the promoter gave the best results, and was chosen for integration into the worm genome. Integration is necessary so that all the cells in the animals contain the construct, thereby preventing loss in some tissues. We used UV light to integrate the construct into the worm genome (see UV Integration protocol in appendix). Several technical problems were encountered with the integration, primarily affecting frequency the frequency of integration. However, we scaled up our effort and obtained two independent, integrated lines. These lines were then assessed for their expression levels. Both lines give similar expression levels and are being further evaluated. Next, we need to incorporate dauer mutations to these *rnr:gfp* strains for our genetic screen. We have chosen *daf-7* and *daf-4*

mutations (null alleles of the ligand and receptor, respectively), since they completely induce dauer formation. Two independent strains have been constructed. We will begin our screen with the daf-4 strain, since this daf-4 allele was used in our microarray experiments described below.

We have generated an alternative construct for detecting cell cycle progression, a *PCNA::gfp* construct. The *PCNA* gene is highly conserved in *C. elegans* and serves as another excellent reporter in vertebrate systems. Unless we find that our *rnr::gfp* construct does not work well in our genetic screen, we will not proceed with developing it further at this time.

Task #2, #3, #4, #6. Perform the genetic screen, characterize the mutants and clone interesting loci. I have grouped these tasks into one section because they all follow logically and experimentally from each other. We had done a small pilot screen early in the project with an existing *rnr::gfp* strain, which proved to be unsatisfactory, prompting us to re-engineer the strain. This pilot screen allowed us to examine some of the screening parameters. This enabled us to evaluate the ease of scoring mutants under a compound microscope (more laborious) vs. a dissecting microscope and to evaluate throughput.

Now that we have successfully integrated our new *rnr::gfp* construct, we will begin a new screen within the next few weeks. A schematic of the genetic screen is shown in the appendix (see Genetic screen scheme). In other work in the lab, we have gained extensive experience on outcrossing mutants, and SNP mapping. SNP mapping has become the method of choice to map mutants to small physical regions of the chromosome, as a prelude to cloning. These techniques require a significant investment of time, and our experience will be valuable in mapping mutants from this screen. Until we have mutants from our proposed screen, the remainder of Tasks #3, #4. #5 cannot be carried out, but we have all the expertise in place. These tasks will move quickly in the next year.

***Task #5.* Do differential hybridizations with DNA microarrays.** As a complementary approach to our genetic screens, our other big aim is to take a molecular approach to find cell cycle genes regulated by the *C. elegans* TGFβ pathway. This task seeks to identify genes that are regulated by a TGFβ induced dauer state. RNA is made from animals just entering the dauer stage and compared to RNA made from non-dauer animals. This RNA is used as a probe to DNA microarrays to determine which genes are induced or repressed. Since the dauer state is a TGFβ induced state, we should be able to identify those genes that connect TGFβ with cell cycle regulation.

We have learned to recognize the phenotypic changes that occur as animals are just entering the dauer state (pharyngeal pumping changes). This enabled us to collect RNA from appropriately staged animals to use in these experiments. Animals were synchronized, and grown under appropriate conditions, and total RNA was made from pools of animals (see RNA protocol in appendix). PolyA RNA was made from total RNA using an Invitrogen FastTract 2.0 mRNA kit. Three independent sets of RNA were generated using these protocols and sent to the Microarray Facility at Stanford

University. At present, commercial microarrays are not available for *C. elegans*, but Dr. Stuart Kim, at Stanford University, operates a free microarray facility for *C. elegans* researchers (funded by NIH). Microarrays containing 17,700 *C. elegans* genes (of 19,000 total) were probed with our RNA, and the results were recently presented to us (see Appendix, page11).

Examining the microarray data is new for most of us in the field and we are in the process of learning how to mine the data in a sophisticated manner. First, one chooses a statistically significant level of RNA expression change. Then, genes are grouped according to increases or decreases in mRNA levels. This results in two groups of genes—those that increase in expression and those that decrease in relation to TGFβ function.

One of the main goals is to identify new genes that connect TGFβ to cell cycle regulation. But to evaluate the robustness of our data, we can examine the status of known cell cycle regulators from these experiments. As expected, we find that some cell cycle genes are more highly expressed, while others are reduced in expression. Most importantly, we find that cyclin D and cyclin E are turned down in these experiments (see Appendix, page 12). Since the animals we picked for mRNA production are just entering dauer, we expect that their cell cycle machinery should be turned down (see a list of known cell cycle gene expression levels in appendix). This independently confirms that our experiment chose the correct animals for analysis. Now we are examining our microarray data to find novel genes whose expression levels change as a result of TGFβ function.

KEY RESEARCH ACCOMPLISHMENTS

1) integrated new *rnr::gfp* constructs into the nematode genome using UV light
2) crossed appropriate genetic markers (*daf-7* and *daf-4*) into integrated nematode strains to prepare for genetic screens
3) completed mRNA preparation from dauered animals in triplicate
4) send mRNA to microarray facility and obtained microarray data from our experiments

REPORTABLE OUTCOMES

We just submitted a paper to the journal Genetics for review, which cites this Department of Defense research grant. Since it is under review, we have not included it in our appendix, but will do so in the final report next year.

CONCLUSIONS

During this past year, we have made substantial progress toward our goals. We have successfully obtained microarray data from the *C. elegans* facility and are in the process of mining the data. We find that indicators of cell cycle progression are turned down in our mRNA samples, supporting the idea that we picked appropriately aged

animals. In other experiments, we have successfully made integrants of our newly improved reporter construct and established appropriate strains for conducting a genetic screen. During the next year, we will continue to mine the volumes of data obtained from the microarray experiments and carry out our genetic screen. At the end of the year, we hope to have some mutants identified and possibly cloned.

REFERENCES

Catzavelos, C., N. Bhattacharya, Y.C. Ung, J.A. Wilson, L. Roncari, C. Sandhu, P. Shaw, H. Yeger, I. Morava-Protzner, L. Kapusta, et al. (1997). Decreased levels of the cell-cycle inhibitor p27Kip1 protein: prognostic implications in primary breast cancer. Nat Med 3, 227-30.

Hong Y., R. Roy, and V. Ambros (1998). Developmental regulation of a cyclin-dependent kinase inhibitor controls postembryonic cell cycle progression in *Caenorhabditis elegans*. Development 125: 3585-3597.

UV Integration

This is a modified version of from Shohei Mitani's WBG 14(1):22. Modifications by Oliver Hobert and Nektarios Tavernarakis

Irradiate 100 L4 animals. You can pick them or use mixed stageplate, and pick L4's after mutagenesis. Having the worms free of bacteria is key. You can pick them to a non-seeded plate, and let them s for a half-hour to remove the layer of bacteria coating them, or wash them in m9.

Irradiate with Stratagene UV crosslinker. Set energy units to 350, machine will automatically deliver that amount and stop. You will see the display count the units as it goes. (Energy delivered is equivalent to 35,000 microjoules, 300 J/m2, and other energy levels with incompatible units. Depends on who you believe). The petri dish should be placed on top of its lid, and on the floor of the machine close to the sensor.

Tip from M. Maduro's web page (see below): "Make sure there is absolutely no E. coli around. I tried several times to do a dose-response curve with food on the plate and got wildly different results each time. I finally got repeatable results when I washed the worms out of all the bacteria and put them down on an unseeded plate. The bacteria absorb the UV – they must act like sunscreen!" (Eric Moss) Comment: This is a great suggestion! I had assumed that as long as the plate lids were removed, the UV treatment itself would be fine. This is an important observation about UV, and may explain why initial attempts were not successful.

"I've used the UV Stratalinker 1800, with the energy setting at 300. Initially I had great successes and got a frequency of about 1% (for 3 or 4 integrants; all rol-6); I have lately, however, had a hard time to get an integrant. Might be the array, though (or the UV lamp getting old)." (O. Hobert, Ruvkun Lab)

Nektarios found that putting the worms at the level of the sensor inside the chamber and a dose of 300 gave tremendous lethality in the F1s, but the survivores had a high integration rate. A dose of 300 with the worms on the chamber floor gave about 40% dead F1 eggs, and a respectable integration rate.

Pick 5 P0 per plate to fresh plates, til you pick 100 P0. Let these plates starve. Oliver waits until they are starved, but has not tested to see if the time of harvest makes any difference. Pick 10-15 rollers from each plate the next day--pick the L3 rollers, i.e., the recovered L1s.

Usually get 2-4 **homozygous** integrants from your 200-300 picks. Oliver recommends being very stringent about categorizing integrants; that is, 100% of the animals should show the transgenic phenotype. 99% is not enough. If you are following Rol for a GFP line, use the GFP rather than Rol to define whether the line is 100% (Rol has incomplete expressivity)

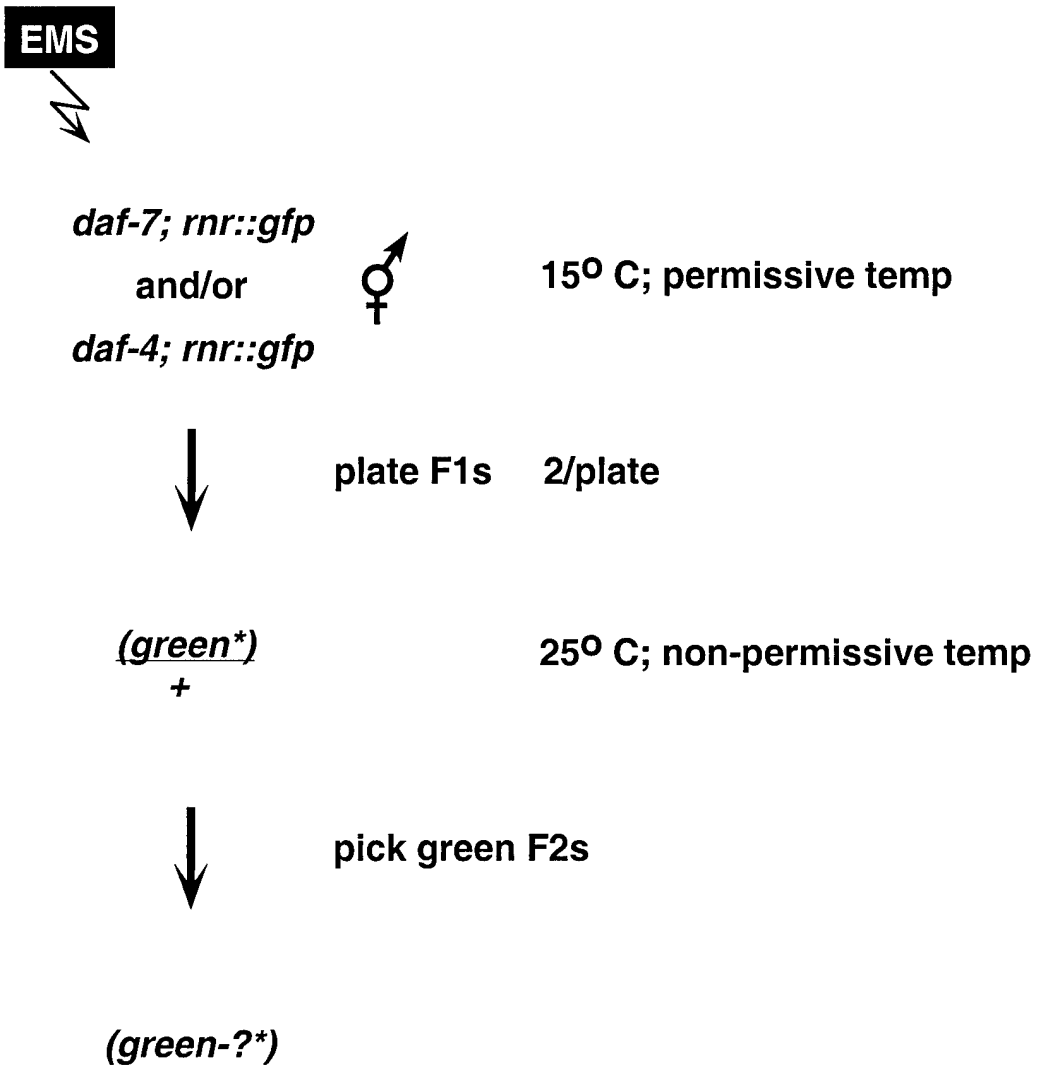
Once you have an putative integrant, carefully repick and score to verify. Also backcross and verify that y can rehomozygose the integrant--Oliver sees fakes that can't be rehomozygosed.

Sometimes the selected marker is more expressed than the unselected marker. e.g., if the array has a GFP and rol-6, selecting integrants for rol-6 will lead to arrays with varying levels of GFP. Some will be high rol, high GFP, some high rol with less GFP. Selecting array based on GFP will produce arrays with strong GFP and variable rol. It is generally not a problem, selecting for rol gives adequate GFP, but it's something to keep in mind.

Daf-c Suppressor Screen

for

Cell Cycle Regulatory Mutants



Total RNA Preparation from C. elegans

harvest worms, spin down, measure the volume of pelleted worms

add trizol—4 ml trizol/1 ml packed worms

vortex vigorously until completely resuspended (longer than 1 min)

flash freeze in liquid N2, thaw at 37°C. vortex. repeat

can store at –80°C at this point

add 2-3 ml more trizol/ml starting packed worms. vortex

add 2 ml CHCl3/ml starting packed worms

shake 15 sec by hand. let sit at RT for 3 min

spin at 12000g, 15 min, 4°C

remove top aqueous layer to fresh 15-ml conical tube

add equal volume isopropanol, mix well. RT 10 min

spin at 12000g, 10 min, 4°C. pour off supernatant

wash pellet in 10 ml 75% EtOH

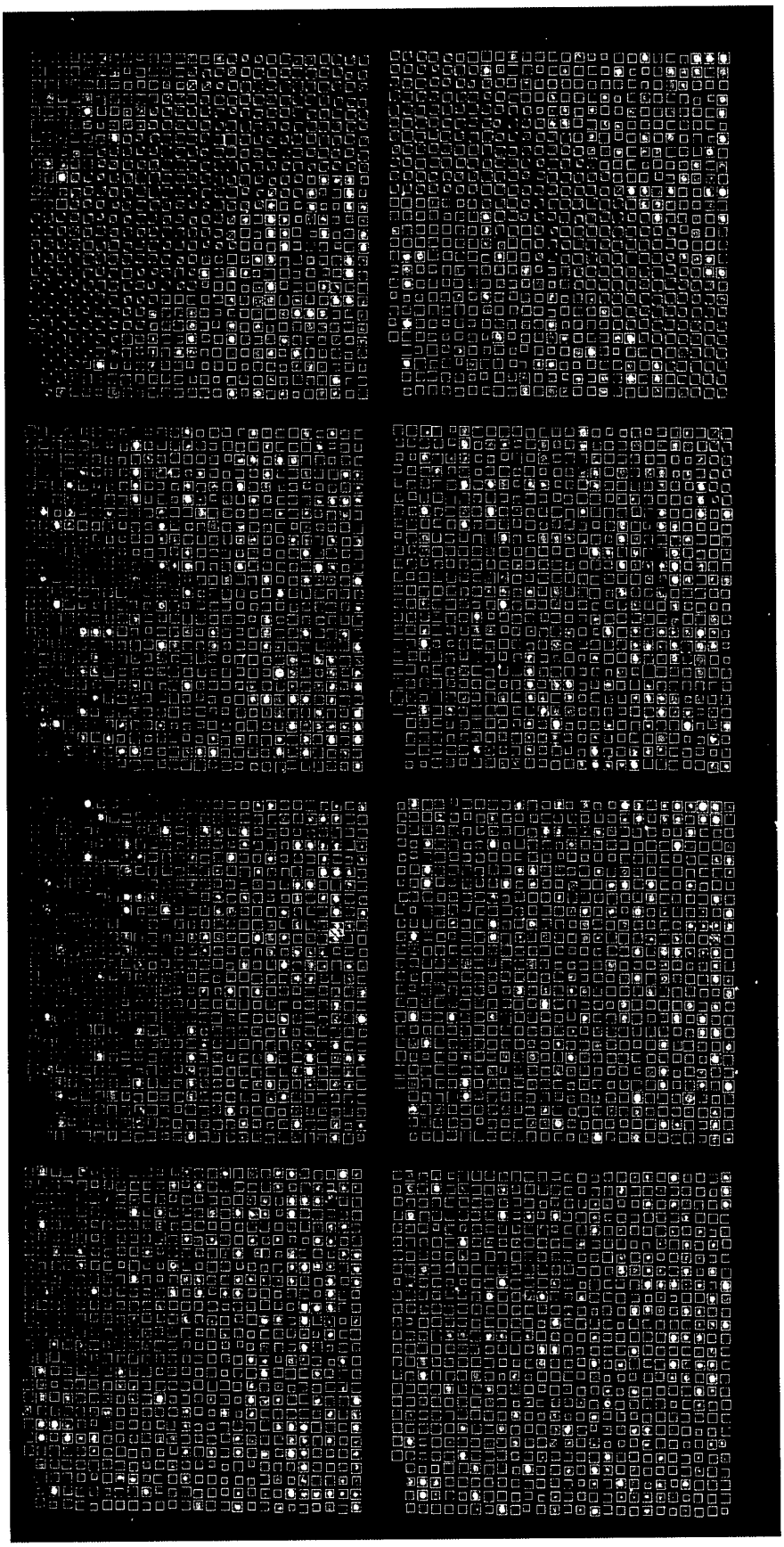
spin at 7500g, 5 min, 4°C. pour off supernatant

do not let pellet dry completely (approx. 5 min air dry)

dissolve in DEPC-ddH2O (about 0.5 to 1 ml per ml of starting packed worms)

take OD at 260 and 280

run 1 ul on 0.8% gel (doesn't have to be RNAse free)



Gene name	directional	upregulated in agreement	Description
F35G12.9	y	?	Protein containing a C3HC4-type (RING finger) zinc finger domain, has similarity to S. cerevisiae cell cycle proteins Apc11p and Hrt1p
C06A1.1	y	Daf-4	Member of the proteasome complex protein family
C07G1.3	y	Daf-4	Serine/threonine protein kinase of the PCTAIRE subfamily of protein kinases, all of which are structurally related to p34cdc2 cyclin-dependent protein kinase
C13G3.3A'	y	Daf-4	Putative B' regulatory subunit of protein serine/threonine phosphatase 2A (PP2A)
C41C4.8	y	Daf-4	Member of the proteasome complex protein family
D2045.6	y	Daf-4	Cell cycle control protein, member of the cullin family of cell cycle control proteins
F43D2.1	y	Daf-4	Putative cyclin, protein with strong similarity to human CCNK protein, a cyclin K
F44B9.3	y	Daf-4	similarity to human CCNT1, cyclin T1
F46A9.5	y	Daf-4	Putative cyclin A/CDK2-associated transcription elongation factor, has strong similarity to H. sapiens TCEB1L gene product
F55B12.3	y	Daf-4	Protein which negatively influences LIN-12 signaling; binds the presenilin homolog SEL-12
R07E4.6	y	Daf-4	Regulatory subunit of cAMP-dependent protein kinase that binds KAP-1
R08C7.2	y	Daf-4	Member of the Cdc50p-like protein family
T05A6.1	y	Daf-4	G1-phase cyclin-dependent kinase inhibitor; member of the CIP/KIP (p21/p27) family of cyclin-dependent kinase inhibitors
T27E9.3	y	Daf-4	Serine/threonine protein kinase with similarity to cyclin-dependent protein kinases
T28B4.1	y	Daf-4	Protein containing an F-box domain, has weak similarity to S. cerevisiae Grr1p, an F-box protein involved in SCF/ubiquitin-mediated degredation of cell cycle regulator
B0261.2	y	N2	Member of the phosphatidylinositol kinase protein family
C37A2.4	y	N2	Putative cyclin E2, has strong similarity to human and D. melanogaster cyclin E proteins, has similarity to S. cerevisiae Clb proteins
C43E11.1(	y	N2	strong similarity to Human CDC18L, cell division cycle 18
C52D10.8	y	N2	similarity to the SKP1 family of proteins, putative paralog of C. elegans C52D10.6 and Y47D7A_138.D
F10B5.6	y	N2	similarity to S. cerevisiae Cdc16p, a component of anaphase-promoting complex (APC) that is required for cyclin degradation and for the metaphase-anaphase transit
F18C5.3	y	N2	strong similarity to human DRIM, which is down-regulated in metastasis
F29B9.6	y	N2	Member of the ubiquitin-conjugating protein family
F32A11.2	y	N2	similarity to S. pombe and human RAD17, cell cycle checkpoint proteins
F32E10.4	y	N2	Member of the karyopherin-alpha protein family
F34D10.2	y	N2	strong similarity at the N-terminal half to human CDC45L protein, cell division cycle 45-like protein
F44B9.4	y	N2	strong similarity to human CCNT1, cyclin T1
F45E12.3	y	N2	Member of the cullin family of cell cycle control proteins
F48E8.7	y	N2	strong similarity to human Hs.159269, a cyclin A/CDK2-associated p45 (Skp2) protein
F53G2.7	y	N2	strong similarity to human MNAT1 (menage a trois 1) a putative Ring finger containing protein, a CDK7-cyclin H complex assembly factor
F54H12.5	y	N2	Member of the F-box domain protein family
F59H6.7	y	N2	similarity to cyclins of human, D. melanogaster and S. cerevisiae, putative paralog of C. elegans F08F1.9
K06H7.5	y	N2	Putative cell cycle control protein, contains a cullin domain, has weak similarity to S. cerevisiae anaphase-promoting complex component APC2, has weak similarity to h
K08E7.7	y	N2	Member of the cullin protein family of cell cycle control proteins
M03F8.3	y	N2	Putative ortholog of Drosophila crooked neck (crn) and S. cerevisiae Syf3p, putative pre-mRNA splicing factor
R03D7.7	y	N2	Protein that functions in development of the germ lineage, has similarity to D. melanogaster nanos
R10E4.4	y	N2	Member of the MCM initiator complex (DNA replication) protein family
T05G5.3	y	N2	Serine/threonine protein kinase, putative ortholog of human CDC2 and S. cerevisiae Cdc28p cyclin-dependent serine/threonine protein kinases involved in cell-cycle r
T06E4.3	y	N2	Putative ortholog of H. sapiens ATR protein (ataxia telangiectasia and Rad3 related) FRAP-related protein
T06E6.2	y	N2	Member of the cyclin B protein family
Y38F1A.5	y	N2	Probably a Cyclin D homolog, may act to control postembryonic G1 progression
Y43E12A.1	y	N2	Member of the cyclin B protein family

Y49F6B.R	y	N2 Putative cyclin H
Y69A2A_2:	y	N2 Small protein with similarity over N-terminal half to human MAD2 and S. cerevisiae Mad2p, a spindle-assembly checkpoint protein
ZC168.4	y	N2 Member of the cyclin protein family
ZK1127.1	y	N2 Protein that functions in development of the germ lineage, has similarity to D. melanogaster nanos
B0285.1	n	Serine/threonine protein kinase with strong similarity to cyclin-dependent protein kinases
C50F4.11	n	Coiled-coil protein with weak similarity to a family of D. melanogaster myosin heavy chains (see BLAST, see SMART), interacts with mdf-2 and is involved mitotic gen
C52D10.6	n	similarity to the SKP1 family of proteins, putative paralog of C. elegans C52D10.8 and C. elegans Y47D7A_138.D
F01G12.6	n	strong similarity to human Hs.179747 protein, a EVI5 homolog, a cell cycle regulator
F02E9.2A	n	Protein required for developmental timing of the ectoderm and cuticle; has weak similarity to H. sapiens YB1 gene product
F10C5.1	n	strong similarity to human CDC23 and S. cerevisiae Cdc23p, a component of the anaphase-promoting complex
T05A6.2	n	G1 phase cyclin-dependent kinase inhibitor; member of the CIP/KIP (p21/p27) family of cyclin-dependent kinase inhibitors
T12C9.4	n	moderate similarity to cyclins of human, S. cerevisiae, and D. melanogaster
T23F11.3	n	strong similarity over C-terminal half to human cyclin-dependent kinase 5 regulatory subunits 1 and 2 (p35/CDK5R1, p39/CDK5R2)
ZK1307.6	n	Putative ortholog of S. cerevisiae Cdh1p and of Drosophila fzr, proteins that are involved in cyclin desctruction
ZK520.4	n	Member of the cullin family of cell cycle control proteins
ZK856.1	n	Member of the cullin family of cell cycle control proteins
cdk-7		
F38H4.9		Member of the protein phosphatase protein family, predicted to be part of the PP2A core complex
H26D21.B		strong similarity to human HUS1 and S. pombe Hus1p cell cycle checkpoint protein
mdf-2		
plc2		
W01A6.E		Member of the cullin family of cell cycle control proteins
Y54G2A.A		